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INTRODUCTION:

Type I diabetes is a metabolic disorder resulting from the destruction of insulin secreting β-cells in the pancreatic islets of Langerhans by T-cell mediated autoimmunity (Eiselein *et al.,* 2004). Insulin therapy is the standard therapy once clinically significant β-cells are destroyed; while pancreas transplantation is an alternative but its application is often limited by availability of donor cells and sustained use of immunosuppressors (Ricordi & Strom, 2004). Pluripotent stem cells (PSCs) can be used for cell-based therapy due to their ability to produce large numbers of insulin-producing β-cells. They can be differentiated into pancreatic lineage *in vitro* using a combination of growth factors and inhibitors that mimic their *in vivo* organogenesis.

Signaling pathways such as FGFs, TGF-β, WNT, Retinoic acid, Hedgehog, and Notch play important roles during pancreatic organogenesis (Kim & Hebrok, 2001; Pan & Wright, 2011; Bastidas-Ponce *et al.,* 2017). However, most of the data surrounding morphogens has been extrapolated from mice and there are no reports of human systems that show a requirement for these pathways. This can be done only using human pluripotent stem cells (hPSCs).

These signaling pathways may regulate the expression of pancreas specific genes via interaction of their downstream effector molecules with epigenetic modulators to establish time-dependent differentiation boundaries or patterning.

One such epigenetic system important in defining cell fate decisions are the Polycomb group (PcG) proteins that function in large multi-protein complexes known as the Polycomb Repressive Complexes (PRC). In mammals, two main Polycomb group complexes exist, Polycomb repressive complex 1 (PRC1) and 2 (PRC2). The Polycomb complexes are key transcriptional repressors silencing many genes crucial for differentiation and cell proliferation. PRCs suppress gene transcription through histone post-translational modifications, altering chromatin accessibility and dynamics ultimately silencing genes whose promoters they occupy. PRC2 consists of three core subunits, EZH1/2, EED, and SUZ12. EZH1/2 catalyze di- and trimethylation of histone H3 lysine 27 (H3K27me3) (Cao *et al.,* 2002; Jani *et al.,* 2019). PRC1 is responsible for ubiquitylation of lysine 119 on histone H2A (H2AK119ub) through its enzymatic core of RING1A or RING1B and PCGF1-6 (Wang *et al.,* 2004; Tamburri *et al.,* 2020). Together, PRC1 and PRC2 mediate chromatin compaction that results in gene silencing.

PcG studies during the differentiation of stem cells towards a pancreatic lineage are of particular interest, as they repress a large number of transcription factors functioning in organogenesis, morphogenesis and pattern specification (Bracken *et al.,* 2006; Kloet *et al.,* 2016). During differentiation into specific lineages, they play an important role by precisely controlling gene expression which has been shown by several studies. Insights into the dynamic mechanisms of PcGs have revealed their role in maintaining the delicate balance between cellular choices faced by multipotent pancreatic progenitors (Xu *et al.,* 2011; van Arensbergen *et al.,* 2012; Xie *et al.,* 2013; Pethe *et al.,* 2014; Xu *et al.,* 2014; Lu *et al.,* 2018). An assessment of the cell choices in mouse foregut endoderm found that Ezh2-mediated H3K27me3 controls the decision between the liver and pancreas decisions (Xu *et al.,* 2011). At the pancreatic progenitor stage, Ring1b marks the transcriptional landscape for repression in terminally differentiated insulin-producing cells (van Arensbergen *et al.,* 2012). Loss of Ring1b in MPCs, but not in mature β-cells, leads to derepression of PcG targets and disrupts insulin secretion in committed cells. Enhancers of key endocrine transcription factors are enriched with H3K27me3 marks and restricts the pool of endocrine progenitors (Xu *et al.,* 2014).

Recent work published in human medulloblastomas, show the effector molecule of the Shh pathway, Gli1 preferentially binds to the *Bmi1* promoter and induces its expression (Wang *et al.,* 2012). However, whether signaling pathways interact with PcG proteins and control their gene repression activity during pancreatic differentiation from human embryonic stem cells (hESCs) remains to be seen. How exactly PcG proteins interact with and are affected by developmental signaling pathways during pancreatic differentiation is not known. No group to our knowledge is focusing on this aspect of PcG regulation of pancreas specific genes in response to developmentally crucial signals such as sonic hedgehog and retinoic acid pathways.

LACUNAE /RATIONALE:

PcG proteins are known to play several crucial roles during pancreas development. During endoderm specification, β-CATENIN along with pluripotency factor OCT4 evicts EZH2-SUZ12 from the promoters of *EOMES*, *SOX17*, *MIXL1*, and *FOXA2* (Xie *et al.,* 2013; Ying *et al.,* 2015). Subsequent reduction of H3K27me3 marks allows induction of the mesendoderm and endoderm lineages. Similarly, depletion of EZH2 from *MIXL*, *T*, and *GSC* by Activin/Nodal-induced SMAD2 allows mesendoderm differentiation of hESCs (Wang *et al.,* 2017). Xu and colleagues found Ezh2 binds regulatory elements of Pdx1 and restrains pancreatic specification in the multipotent gut cells (Xu *et al.,* 2011). Such studies revealed that PcG proteins are key modulators of cell fate choices. However, less is known about roles of PcG proteins at intermediatory stages of pancreatic differentiation. Also, most studies have focused on PcG-mediated catalyzed histone modifications such as marks, H3K27me2/3 and H2AK119ub1. However, enrichment of Polycomb subunits at regulatory elements have been shown to affect chromatin compaction outside of their histone post-translational modifications.

Although nearly all pancreatic differentiation protocols incorporate SHH pathway antagonists and retinoic acid agonists, their underlying role during hESC differentiation into pancreatic lineage specification remains largely unexplored (D'Amour *et al.,* 2006; Pagliuca *et al.,* 2014; Rezania *et al.,* 2014; Nostro *et al.,* 2015; Hogrebe *et al.,* 2020). Several studies have shown that epigenetic regulators can respond to signaling pathways and bring about changes in cell specification. Retinoid regulation of gene expression often involves interaction with PcG proteins. In mouse embryonic stem cells (mESCs), RA activates transcription of its primary targets through the removal of Ezh2/PRC2 (Kashyap *et al.,* 2011). Previous studies in multiple myelomas, pancreatic cancer, medulloblastoma, and breast cancers show active Shh signals leads to an overexpression of PRC1 subunit, Bmi1. Overexpression of Bmi1, oncogene promotes extensive proliferation of the cancer cells. In human medulloblastomas, Shh directly activates *Bmi1* through the effector molecule of the sonic hedgehog pathway, Gli1 (Leung *et al.,* 2004; Liu *et al.,* 2006; Wang *et al.,* 2012). Although current literature implicates *Bmi1* as a direct downstream target of Shh signaling in cancer cells, whether Shh ligands can regulate Bmi1 during the course of normal pancreatic differentiation has not been elucidated yet. It has not been demonstrated whether these signaling pathways regulate histone modifiers, especially PcG proteins to bind and control expression of key pancreas specific genes. Another limiting factor is the fact that

most of this data comes from mouse models. Since human development cannot obviously be manipulated in the same way we do when experimenting on a mouse or a fruit fly, much of our knowledge about human development is extrapolated from studies of model organisms. Pancreatic cells derived from hESCs and the differentiation system of pancreatic intermediates serve as good models to elucidate the roles of transcription factors, epigenetic states, chromatin modulators, and noncoding RNAs during pancreatic development (Gaertner *et al*., 2019).

The aim of our study was to investigate the roles of SHH and RA on PcG proteins during pancreas development. For this, the pathways would be modulated using small molecules and study the effect of this modulation on the differentiation outcome. Our primary focus was whether external signaling morphogens such as SHH and RA could direct cell-intrinsic epigenetic pathways involving PcG proteins, and to see whether these molecular associations control pancreatic cell fate programs. We also wanted to understand the localization of PRC1 upon pathway modulation at pancreas specific-genes during the step-wise differentiation from human embryonic stem cells.

AIM

To study the role of sonic hedgehog and retinoic acid signaling pathways in regulation of lineage specific genes via Polycomb Group (PcG) proteins during human pluripotent stem cells (hESCs/hiPSCs) differentiation into pancreatic lineage.

Objectives:

- **i.** To differentiate human pluripotent stem cells (hESCs/hiPSCs) into pancreatic lineage and their characterization.
- **ii.** To analyze the effect of modulation of sonic hedgehog and retinoic acid pathways on pancreatic differentiation and expression of Polycomb group proteins.
- **iii.** To determine promoter occupancy of Polycomb group proteins at key pancreas specific genes in response to modulation of sonic hedgehog and retinoic acid pathways.

MATERIAL AND METHODS:

i) **Human embryonic stem cell (hESC) culture –** Human embryonic stem cell line, KIND1 was provided by Dr. Deepa Bhartiya from the National Institute for Research in Reproductive Health, Mumbai. Human embryonic stem cell culture research was approved by the SVKM's Institutional Committee for Stem Cell Research (ICSCR), NMIMS (Deemed-to-be) University, Mumbai, India.

hESC line, KIND1 was successfully maintained, cryopreserved, and revived over several passages. The undifferentiated stem cells were maintained in xeno- and feeder-free conditions on Vitronectin (Gibco, MA, USA) (0.5μg/cm^2/RT/1 hour, prior to use) coated cell culture dishes in Essential 8 basal media (Life Technologies, CA, USA) supplemented with 1X Essential 8 supplement (Life Technologies). Cultures were passaged at a split ratio of 1:3-1:4 every four days using 0.5 mM EDTA (Sigma-Aldrich, MO, USA). The cells were cryopreserved in liquid nitrogen using dimethyl sulfoxide (DMSO) at final concentration 10% as the cryoprotectant.

ii) **Directed differentiation –** Directed differentiation of KIND1 cells towards a pancreatic lineage was carried out as described previously (D'Amour *et al.,* 2006; Pethe, Nagvenkar & Bhartiya, 2014), with some minor modifications. Undifferentiated KIND1 cells showing ~75%–80% confluency were used for differentiation and harvested at days 0, 4, 8, 12, and 16 for protein and RNA extraction. Following are the conditions under which directed pancreatic differentiation (termed control) was performed. Briefly, cells were rinsed with DPBS followed by culturing in RPMI-1640 (Sigma-Aldrich) media supplemented only with 0.025× ITS (Sigma-Aldrich), 100 ng/ml ACTIVIN A (R&D Systems), and 25 ng/ml WNT-3A (R&D Systems) for the first day and an additional three days in RPMI-1640 containing 100 ng/ml ACTIVIN A and 2% fetal bovine serum. From day 4 onwards, cells were cultured in DMEM/F-12 (Thermo Fisher Scientific, MA, USA) containing 2 μM all-trans-retinoic acid (Sigma-Aldrich), 100 nM LDN193189 (Sigma-Aldrich), 20 ng/ml FGF4 (Life Technologies, CA, USA), and 1× B-27 supplement (Gibco). Between days 8 to 12, cells were cultured in DMEM/F-12 containing 2 μM all-trans-retinoic acid, 100 nM LDN193189, 25 ng/ml FGF10 (Life Technologies), 1× B-27 supplement, 1× Glutamax (Gibco), and 1× non-essential amino acids (Gibco). In the last stage of differentiation (day 12–16), cells were cultured in DMEM/F-12 containing 2 μM all-trans-retinoic acid, 100 nM LDN193189, 25 ng/ml FGF10, 1× B-27 supplement, 1× Glutamax, and 1× non-essential amino acids.

Between days 4 to 12, modulation of the sonic hedgehog pathway was carried out. 0.25 µM SANT1 (Sigma-Aldrich) was added to the culture media and will be referred to as 'SANT1' studies, antagonist from hereon while cells cultured with 25 ng/ml of recombinant human SONIC HEDGEHOG (Sigma-Aldrich) will be referred to as 'SHH' (agonist) studies. Similarly, the retinoic acid pathway was modulated between days 4 to 12 using pathway antagonist, 1 µM LE135 (Sigma-Aldrich) and were termed as 'LE' studies. All-trans RETINOIC ACID was added to the culture media at a concentration of 2 µM and termed as 'RA' studies. Control groups were differentiated without exogenous antagonists or agonists but were exposed to the same quantity of vehicle.

iii) **Immunofluorescence staining** – Cultures were fixed with 4% paraformaldehyde in 1x PBS for 10 minutes /room temperature and washed four times with 1x PBS. Non-specific proteins were blocked using 2% bovine serum albumin (in 1x PBS) for 2 hours /room temperature followed by incubation with primary antibody solutions overnight at 4°C. After washing off excess antibody with 1x PBS containing 0.02% vol/vol Tween-20, cells were incubated with Alexa-conjugated secondary antibodies for 2 hours /room temperature. Primary and secondary antibody solutions were prepared in 1x PBS containing 0.5% bovine serum albumin. Primary antibodies used were: rabbit anti-OCT4 (Cell Signaling Technology, MA, USA), rabbit anti-CXCR4 (Abcam, Cambridge, UK), and rabbit anti-PDX1 (Abcam). Post-incubation with secondary antibody (goat anti-Rabbit IgG Alexa Fluor 488, Thermo Fisher Scientific), cells were washed with 1x PBS containing 0.02% vol/vol Tween-20 and counterstained with DAPI (Invitrogen, MA, USA) at a final concentration of 300 nM for 5 minutes /room temperature. Images were captured at 10X, 20X or 40X magnifications using the Axio Vert.A1 software. Image analyses were performed using ImageJ software (NIH) (http://rsbweb.nih.gov/ij/).

iv) **Protein extraction –** Homogenization of cells was carried out in a cell lysis buffer [50 mM Tris, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 0.1% SDS, 0.1% Na-deoxycholate, 1% Triton X-100 and 1x Protease Inhibitor Cocktail (Amresco, OH, USA)] for 30 minutes on ice to obtain total protein. The lysate was clarified at 13,000 g for 20 minutes at 4°C. Total histone extraction from cells was performed as described by de Napoles et al. (2004) with some modifications. Harvested cells were subjected to rapid cycles of flash-freezing and thawing for 20 minutes before resuspension in 1x PBS containing 1x Protease Inhibitor Cocktail (Amresco) and 1 mM PMSF. Cell suspensions were incubated on ice for 15

minutes, followed by centrifugation at 250 g for 4 minutes at 4°C. Histone proteins were extracted by dissolving the pellets in 500 µL of ice-cold 0.2 M H_2SO_4 and incubated on ice for 30 minutes. The supernatant was separated by centrifuging at 20,200 g for 20 minutes, and 4°C and histones were precipitated using 6.1 N tricholoroacetic acid overnight at -20°C. Histones were pelleted by centrifuging at 20,200 g for 10 minutes at 4°C and washed twice with 500 µL ice-cold 100% acetone for 10 minutes on ice. The histone extracts were clarified at 20,200 g for 10 minutes at 4°C. Pellets were air-dried and reconstituted in 100 mM Tris buffer (pH 7.2 - 7.4). Protein concentrations were measured by the Lowry assay using Folin-Ciocalteu's phenol reagent (Sigma-Aldrich). 5-10 µg of histone proteins were fractionated by 15% SDS-polyacrylamide gels and immunoblotted.

Protein concentrations were measured by the Lowry assay using Folin-Ciocalteu's phenol reagent (Sigma-Aldrich).

v) **Immunoblot Analysis –** Undifferentiated and differentiated cells were rinsed and scraped into ice-cold 1x PBS. Cells were sedimented at 3000 g for 10 minutes at 4°C before lysis in a cell lysis buffer and 1x Protease Inhibitor Cocktail (Amresco) for 30 minutes on ice. Equal amount of protein (15-20 µg) was loaded on 10-15% SDS-polyacrylamide gels, resolved by electrophoresis and electroblotted onto 0.45 µM polyvinylidene difluoride membrane (Amersham). Membranes were blocked with 5% non-fat dairy milk/TBST followed by overnight incubation at 4°C with primary antibody dilutions prepared in 3% non-fat dairy milk/TBST. The following primary antibodies were used: rabbit anti-OCT4 (Cell Signaling Technology, 2840; 1:1000), rabbit anti-BMI1 (Cell Signaling Technology, 6964; 1:1000), rabbit anti-RING1B (Abcam, ab101273; 1:2000), rabbit anti-PDX1 (ABclonal Science, Wuhan, China, A10173, 1:700), rabbit anti-PTCH1 (ABclonal Science, A0826, 1:700), rabbit anti-SHH (ABclonal Science, A12503, 1:1000), rabbit anti-SMO (Abcam, ab72130, 1:2000), rabbit anti-GLI1 (Abcam, ab134906, 1:1000), rabbit anti-Histone H2A (Cell Signaling Technology, 12349, 1:1000) rabbit anti-H2AK119ub1 (Cell Signaling Technology, 8240, 1:2000), and mouse anti-β-Actin (Sigma-Aldrich, A5441, 1:5000). Secondary antibodies conjugated to horse-radish peroxidase [anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (Thermo Fisher Scientific) or anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (Genei, Bangalore, India)] were used which helped in detection by enhanced chemiluminescence technique (Bio-Rad Laboratories, CA, USA). Detection was performed using ECL Kit (Bio-Rad Laboratories, Hercules, CA, USA) and imaged in ChemiDoc Gel Documentation system (Bio-Rad Laboratories).

Densitometric analysis was done using ImageJ software (http://rsbweb.nih.gov/ij/) from experiments performed in triplicates and β-ACTIN or total histone H2A was used for normalizing protein levels across samples.

vi) **Total RNA Extraction –** Undifferentiated and differentiated cells harvested at various stages were rinsed with 1x PBS and lysed directly using TRI reagent (Sigma-Aldrich). Total RNA was extracted according to the manufacturer's instructions. Briefly, all samples were lysed in 1 mL TRI reagent and incubated for 10 minutes /room temperature, with intermittent vortexing to aid lysis. 200 µL of chloroform was added for phase separation and shook vigorously for 30 seconds before allowing the sample to stand for 2 minutes /room temperature. Tubes were centrifuged at 12,000 g for 15 minutes /4°C before transferring the aqueous phase into fresh tubes. 500 µL of 2-propanol per mL of TRI reagent was added for RNA precipitation. Samples were incubated overnight at -20°C followed by centrifugation at 12,000 g for 10 minutes /4°C. Pellets were washed twice with 500 µL of 75% ethanol, followed by a final wash with 500 µL of 100% ethanol before air-drying and reconstituting in 30-40 µL of DEPC water. RNA concentrations and purity-check was performed using the Ultraviolet-Visible spectrophotometer (Perkin Elmer, MA, USA). Samples showing a A_{260}/A_{280} ratio >1.8 were subsequently used for cDNA synthesis.

vii) **cDNA Synthesis –** 1 µg of total RNA was subjected to reverse transcription for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad Laboratories). Reaction setup and protocol were according to the manufacturer's instructions. Briefly, 20 µL total volume reaction per sample was setup and incubated in the thermal cycler (Applied Biosystems, MA, USA) with the following conditions: priming at 25°C /5 minutes, reverse transcription at 46°C /20 minutes followed by RT inactivation at 95°C /1 minute.

viii) **Reverse-Transcription PCR –** All RT-PCR assays were carried using the thermal cycler (Applied Biosystems) in combination with the 2X EmeraldAmp GT PCR Master Mix (Takara Bio, Japan). Target-specific primers were designed using NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and efficiency was checked using the cDNA serial dilutions (10-fold) and a standard curve method. Only primers showing an efficiency between 90-110% were used for gene expression studies. The following thermal cycler conditions were used for 10 or 20 µL total volume reactions: initial denaturation at 94°C /3 minutes followed by 35 cycles of denaturation at 94°C /30 seconds,

annealing at 55-61°C /30 seconds and elongation at 72°C /30 seconds. At the end of the amplification phase, PCR products were separated on 2% agarose (MP Biomedicals, CA, USA) containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich). Product sizes were approximated by inclusion of a 100 bp DNA ladder (Thermo Fisher Scientific). Images were captured on a molecular imager (Bio-Rad Laboratories). All measurements were normalized with the housekeeping *GAPDH*.

ix) Real-Time Quantitative PCR – All real-time assays were carried out in duplicates, using the StepOnePlus Real-Time PCR Systems (Applied Biosystems) in combination with PowerUp SYBR Green Master Mix (Applied Biosystems). Target-specific primers were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software and efficiency was checked using the cDNA serial dilutions (10-fold) and a standard curve method. Only primers showing an efficiency between 90-110% were used for gene expression studies. The following thermal cycler conditions were used for 10 µL total volume reactions: initial denaturation at 95°C /2 minutes followed by 40 cycles of denaturation at 95°C /15 seconds, gradient annealing at 55-61°C /15 seconds and elongation at 72°C /1 minute. At the end of the amplification phase, a dissociation step was performed to identify amplicon homogeneity. Relative quantitation of targets within the different samples was determined using the comparative C_T (ΔΔCT) method. All measurements were normalized with the housekeeping *18SrRNA*. In most studies, the expression of the targets in the undifferentiated/D0 sample was used as the calibrator or reference for calculating relative fold change. Thus, graphs represent fold change in expression over that detected in undifferentiated hESCs (shown as 1.0). C_T expression shown is relative to highest detectable (C_T40) in cases when no expression was seen.

x) Chromatin Immunoprecipitation coupled with qPCR – ChIP experiments were performed essentially as described by Bertani et al. (2008) with certain modifications for undifferentiated human hESCs KIND1, and progenitors differentiated during a modulated SHH pathway. Briefly, cells were rinsed with 1x PBS and crosslinked with 1% formaldehyde (Sigma-Aldrich) solution for 10 minutes at room temperature on a rotor. Excess formaldehyde was quenched using 0.125M glycine for 10 minutes at room temperature. Cells were washed with chilled 1X PBS and lysed in lysis buffer [50 mM HEPES-KOH, 1 mM EDTA, 140 mM NaCl, 0.1% SDS, 1% Triton X-100, 1mM PMSF and 1x Protease Inhibitor Cocktail (Amresco)]. Chromatin was sheared using a probe sonicator

(QSonica, Connecticut, USA) to obtain fragments in the range of 150-500 bp and clarified at 20,000 g for 10 minutes at 4°C to pellet insoluble material. [Note: Number of sonication cycles was adjusted based on the number of cells at start. Since the number of cells varied significantly between undifferentiated stage to various days of differentiation, with later days showing higher confluency, number of sonication cycles was adjusted accordingly]. After preserving 1% of the supernatant as input samples, 30 µg of chromatin were incubated overnight at 4°C with 5-10 µg rabbit anti-Bmi1 (Cell Signaling Technology, 6964), rabbit anti-RING1B (Abcam, ab101273) or rabbit anti-H2AK119ub1 (Cell Signaling Technology, 8240) conjugated to Dynabeads Protein A (Thermo Fisher Scientific) according to according to the manufacturer's instructions. Negative control antibody of normal rabbit IgG (Abcam) was included for each sample. Immunoprecipitated complexes were retrieved and washed successively in buffers containing low salts [50 mM HEPES-KOH, 1 mM EDTA, 500 mM NaCl, 0.1% SDS and 1% Triton X-100], high salts [10 mM Tris, 250 mM lithium chloride, 1 mM EDTA, 0.5% sodium desoxycholate and 0.5% NP-40] and Tris EDTA [10 mM Tris and 1 mM EDTA] before eluting target antigen in ChIP elution buffer [10 mM Tris, 1 mM EDTA and 1% SDS]. DNA was further incubated at 65°C/ overnight to reverse crosslinking followed by RNase A (Sigma-Aldrich) (37°C/ 2 hours) and proteinase K (Sigma-Aldrich) (55°C/ 2 hours) treatment. DNA extraction was performed using phenol:chloroform:isoamyl alcohol (Sigma-Aldrich). 400 µL of phenol:chloroform:isoamyl alcohol was added to sample and vigorously shaken for 30 seconds before allowed to stand for 2 minutes /room temperature. Tubes were centrifuged at 20,000 g for 20 minutes /4°C before transferring the aqueous phase into fresh tubes. 800 µL of chilled 100% ethanol was added for DNA precipitation. Samples were incubated overnight at -20°C followed by centrifugation at 20,000 g for 10 minutes /4°C. Pellets were washed once with 500 µL of 80% ethanol, before air-drying and reconstituting in 30-40 µL of Tris buffer. DNA concentrations and purity-check was performed using the Ultraviolet-Visible spectrophotometer (Perkin Elmer).

100 ng of the purified DNA was used as template for amplification on the StepOnePlus Real-Time PCR system (Applied Biosystems) using PowerUp SYBR Green Master Mix reagent (Applied Biosystems). Target-specific primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software and efficiency was checked using the cDNA serial dilutions (10-fold) and a standard curve method. Only primers showing an efficiency between 90-110% were used. Each reaction consisted of 1× SYBR green mix, 50 nM of each forward and reverse primer and 2.5 µL of gDNA template for a

final volume of 20 µL. Cycling parameters were as follows: initial denaturation at 95°C /2 minutes followed by 50 cycles of denaturation at 95°C /15 seconds, gradient annealing at 55-61°C /15 seconds, and elongation at 72°C /15 seconds. At the end of the amplification phase, a dissociation step was performed to identify a single melting temperature for each primer set. Fold enrichment for all data were obtained from biological duplicates with qPCR performed in triple independent amplifications and signals were normalized relative to input DNA.

In the present study, we have used the percent input method for normalization of data. With this method, signals obtained from the ChIP are divided by signals obtained from an input sample. This input sample represents the amount of chromatin used in the ChIP process. Signals from samples (C_T) were divided by signals (C_T) from the input samples taken early during the ChIP procedure to normalize. The input sample indicates the total amount of chromatin used for the ChIP, in our case 10% of the starting volume. [Input DNA is defined as the aliquot of sheared chromatin prior to pull down and is used to normalisation]. C_T value of the input sample is adjusted by subtraction of the dilution factor used (10) represented as logarithmic quantification cycles. Thus, the data represents promoter occupancy with respect to the normalized input Ct. Error bars denote mean ± s.e.m. from three technical repeats of three independent biological studies each (n=3). P values computed using Student's t-test. Asterisks denote *P* values and represent significance between SANT1/SHH treatments at specific time points /stages.

.

RESULTS:

i. **Human embryonic stem cell characterization -** KIND1 cells were cultured in Essential 8 medium on Vitronectin coated dishes and were successfully maintained, cryopreserved, and revived over several passages.

Prior to differentiation of the cells, KIND1 cells were characterized by various assays and the pluripotent nature was studied at the transcript level by RT-PCR for expression of *OCT4*, *NANOG* and *SOX2*. Expression of the key pluripotency marker, OCT4 was also seen at the protein levels (through several passages) by immunoblotting. Localization of this marker within cell nuclei was also seen by immunofluorescence. Karyotyping of cells was performed prior to differentiation to check for any gross abnormalities in the chromosomes due to maintenance in culture. No gross changes in the karyogram were seen (see Figure 1).

Pluripotency of the KIND1 cells was also determined through the embryoid body (EB) formation assay using the hanging drop method. EBs were cultured on gelatin coated dishes for 7 and 14 days and cells were harvested for total RNA extraction. Expression of transcription factors specific for ectoderm (*SOX1*, *PAX6*), mesoderm (*BRCHRY*, *TBX5*), and endoderm (*FOXA2*, *SOX17*) were checked by RT-PCR. Presence of these markers suggested that the cells can spontaneously differentiate and give rise to cells of the ecto-, endo-, and mesoderm lineages.

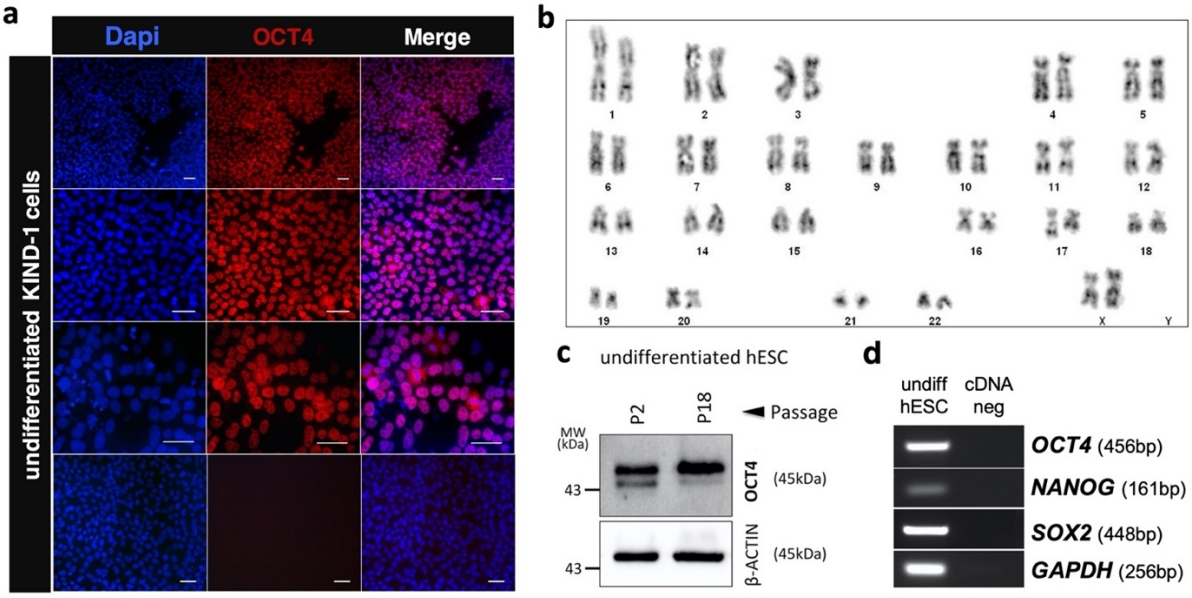


Figure 1: Characterization of the undifferentiated KIND1 cells prior to differentiation.
(**a**) Immunofluorescence analysis for pluripotency marker, OCT4 (Merck, MABD76, 1:250) in hESCs. Scale bar, 50 µm.
(**b**) Immunoblot of anti-OCT4 in undifferentiated cells at various passages.

(**c**) Reverse-transcription PCR showing presence of pluripotency markers *OCT4*, *NANOG*, and *SOX2* in KIND1 cells.

ii. Directed differentiation and characterization of hESC-derived pancreatic progenitors

- Stage 1 involved exposing the cells to high levels of ACTIVIN A that pushed pluripotent stem cells towards an endoderm fate forming definitive endoderm. Definite endoderm (DE) specific transcription factors *CXCR4*, *FOXA2*, and *SOX17* were expressed maximally at day 4, indicating transition towards a DE-like stage. Upregulation of surface-marker, CXCR4 was also confirmed by immunofluorescence wherein most day 4 cells expressed the protein. The definitive endoderm stage is a primary requirement for pancreatic specification and high numbers of DE-like cells enhance further downstream differentiation towards pancreatic lineage. Addition of Retinoic acid, Noggin/ LDN193189, and FGF4 patterned the definitive endoderm cells towards the next stage of differentiation, the primitive gut tube (PG) stage. A 150-fold increase in the *HNF4α* mRNA levels and 50-fold increase in *HNF1B* mRNA levels were indicative of this stage. Continued addition of Retinoic acid, Noggin/ LDN193189, and FGF10 pushed the primitive gut tube cells towards a posterior foregut (PF) fate. Addition of supplements NEAA, Glutamax and B27 increases cell growth and viability. Expression levels of stage-specific *PDX1* and *SOX9* were investigated in day 12 samples using qRT-PCR. *SOX9* showed a dramatic increase at day 8 with minimal expression in definitive endoderm cells, and its expression remained high through day 12. *PDX1* was seen at varying levels in differentiated samples between days 8 to 16 The presence of PDX1 was also confirmed by immunofluorescence wherein 90-95% of the cells stained positive for PDX1 (semi-quantitated using ImageJ software). During pancreatic endoderm (PE) stage formation, between days 12 to 16, expression of *PTF1A* and *PAX6* was seen to increase and expression of these markers was retained by the PE cells. Overall, the presence of specific markers at each of the stages indicated successful differentiation of human embryonic stem cells KIND1 towards a pancreatic fate.

Pluripotent human embryonic stem cells were effectively differentiated into pancreatic lineage (see Figure 2).

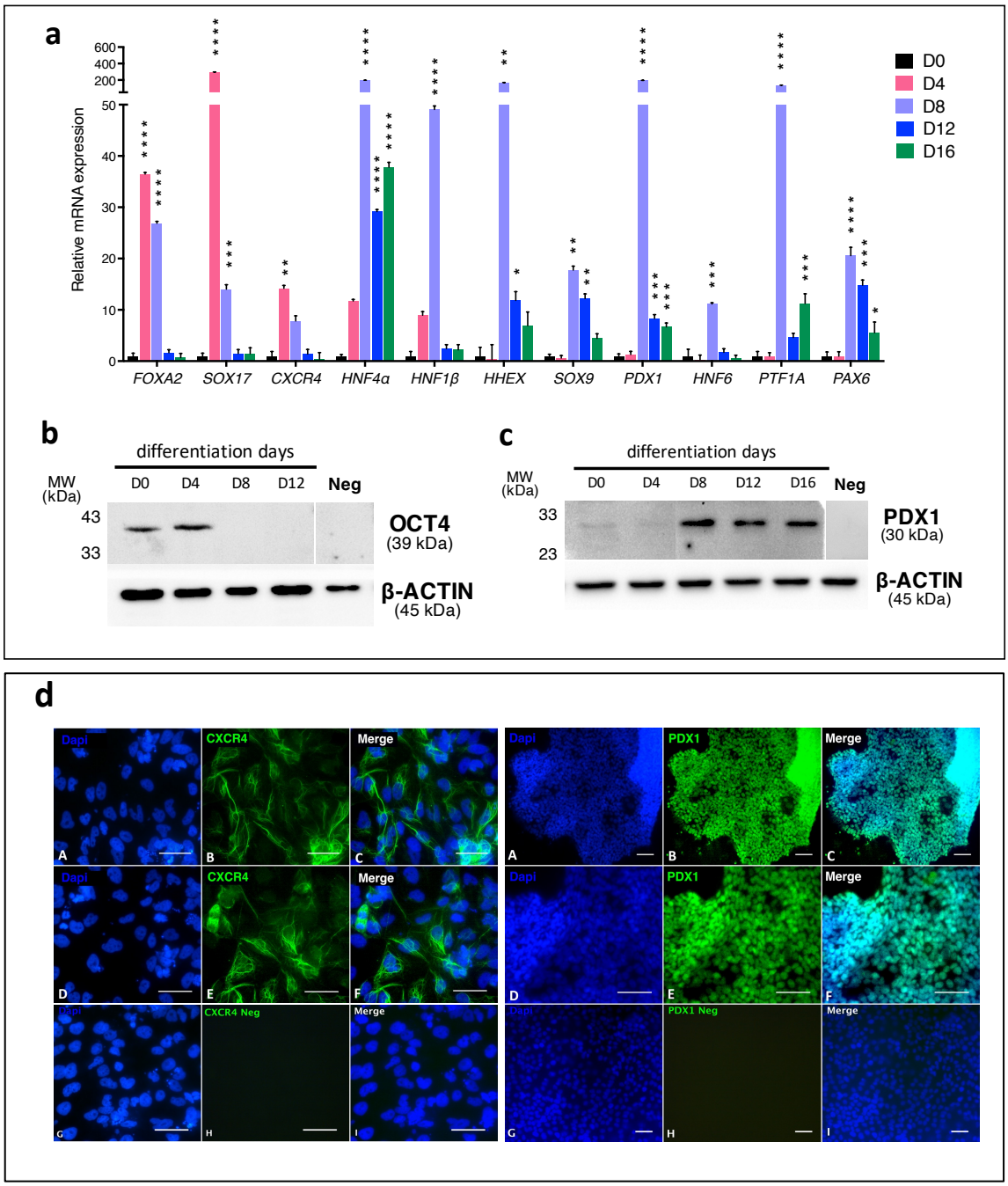


Figure 2: Characterization of hESC-derived pancreatic progenitors.

(**a**) qRT-PCR analyses of pancreatic markers indicating transition towards a pancreatic lineage. mRNA levels were normalized to house-keeping, 18S rRNA and expression was plotted relative to levels in undifferentiated cells. Error bars denote mean±s.d from three technical repeats of three independent biological studies each (n=3). *P* values computed using Student's t-test. Asterisks denote *P* values and represent significance between expression at undifferentiated stage (D0) and the specific stage/ time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

(**b**) Immunoblot analysis of OCT4 (anti-OCT4, Millipore, MABD76) in directed differentiated cells on day 4 (D4), day 8 (D8), and day 12 (D12).

(**c**) Immunoblot analysis showing PDX1 expression in the differentiated cells.

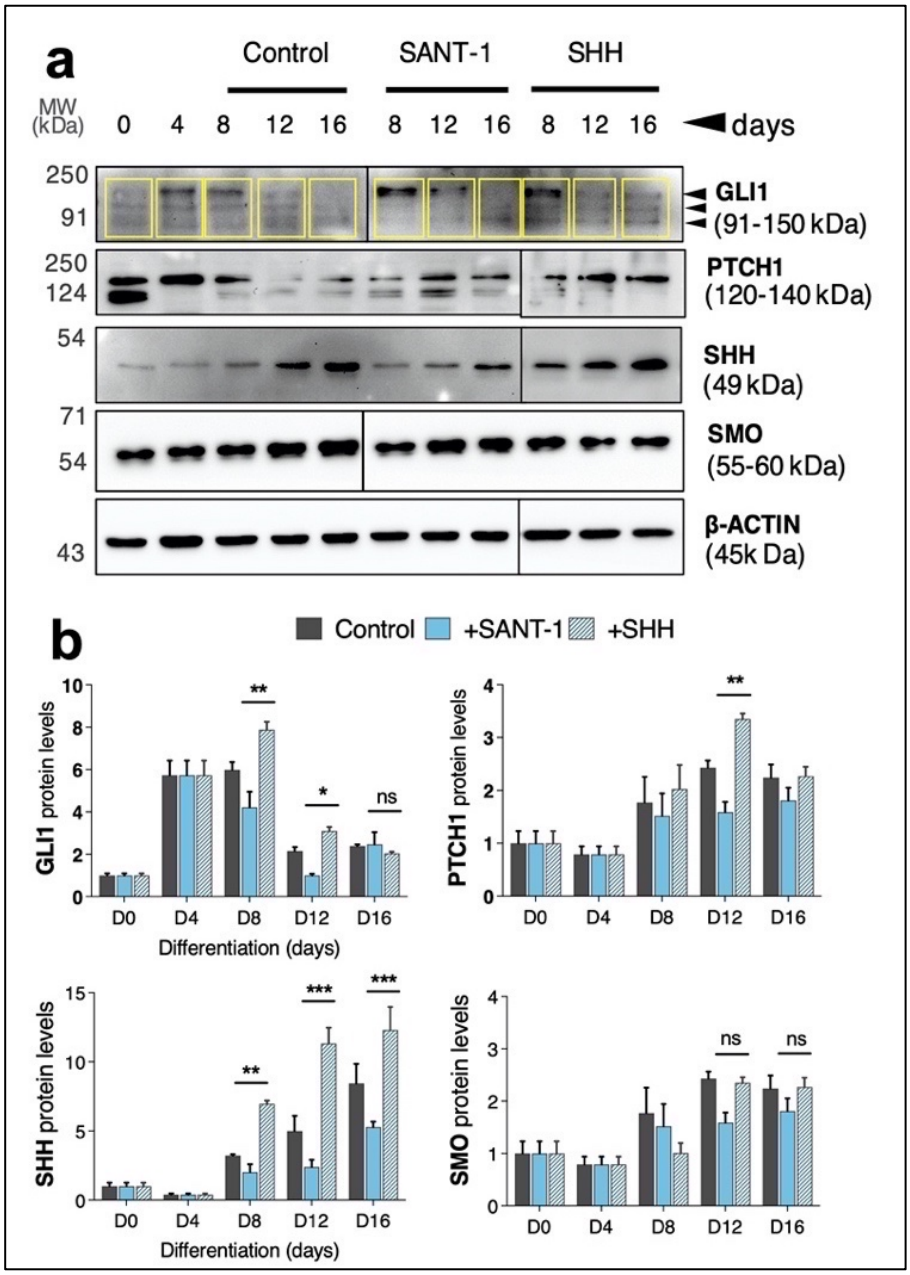
(**d**) Immunofluorescence analysis of DE marker, CXCR4 (Abcam, ab124824) in day 4 cells. Scale bar, 50 µm. Left panel: Immunofluorescence analysis of PE marker, PDX1 (Abcam, 134150) seen within most cell nuclei at day 16. Scale bar, 50 µm.

iii. **Modulation of the sonic hedgehog and retinoic acid pathways *in vitro* -** We differentiated KIND1 cells in the presence of SHH pathway modulators (SANT1 and/or SHH) and RA pathway modulators (LE135 and/or all-trans RA) and evaluated their effects on pancreatic progenitor cell fates. A control group wherein differentiation was carried out in the absence of both small molecules was also maintained to take into account any endogenous levels of SHH-modulators that maybe secreted by the cells.

We analyzed levels of SHH pathway components *PATCHED1* (*PTCH1*), *SMOOTHENED* (*SMO*), and *GLI1* in cells differentiated in the presence of exogenous SHH as well as the inhibitor SANT1. Expression of genes *PTCH1* and *GLI1*, that are transcriptional targets of the pathway were found to be significantly different between the treated groups. Addition of recombinant SHH was accompanied by several fold upregulation in *PTCH1* transcript levels. Stimulation of the pathway also increased expression of the downstream target GLI1, indicating that exogenously added SHH can act as an agonist and activate target gene expression in the hESCs during pancreatic differentiation. The addition of an agonist or antagonist had little appreciable effect on global levels of *SMO*. These results were also confirmed by immunoblot studies. GLI1, PTCH1, and SHH expression was upregulated at days 8 and 12 of differentiation when the SHH pathway was activated in the progenitors. Corresponding to the transcript data, no significant alterations were observed in global SMO protein levels.

Similarly, RA modulation altered expression of retinoic acid receptors *RARα* and *RARβ* (targets induced by RA) in the hESC-derived pancreatic progenitors. Transcript levels of *RARα* and *RARβ* were upregulated several-folds at day 8 and 12 when the cells were differentiated in the presence of all-trans RETINOIC ACID. Expression of these genes remained low in cells treated with RA-agonist, LE135. This indicated that RA signals were regulated through the use of these small molecules in the progenitors.

Transcriptional regulation of these targets not only show successful modulation but also indicated that the pancreatic progenitors can respond to SHH and RA modulation (see Figure 3).


a
Control
SANT-1
SHH
MW (kDa)
0 4 8 12 16 8 12 16 8 12 16
days
250
91
GLI1 (91-150 kDa)
250
124
PTCH1 (120-140 kDa)
54
SHH (49 kDa)
71
54
SMO (55-60 kDa)
43
β-ACTIN (45k Da)
b
Control
+SANT-1
+SHH
GLI1 protein levels
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SHH protein levels
SMO protein levels
D0 D4 D8 D12 D16
Differentiation (days)
**
*
ns
***

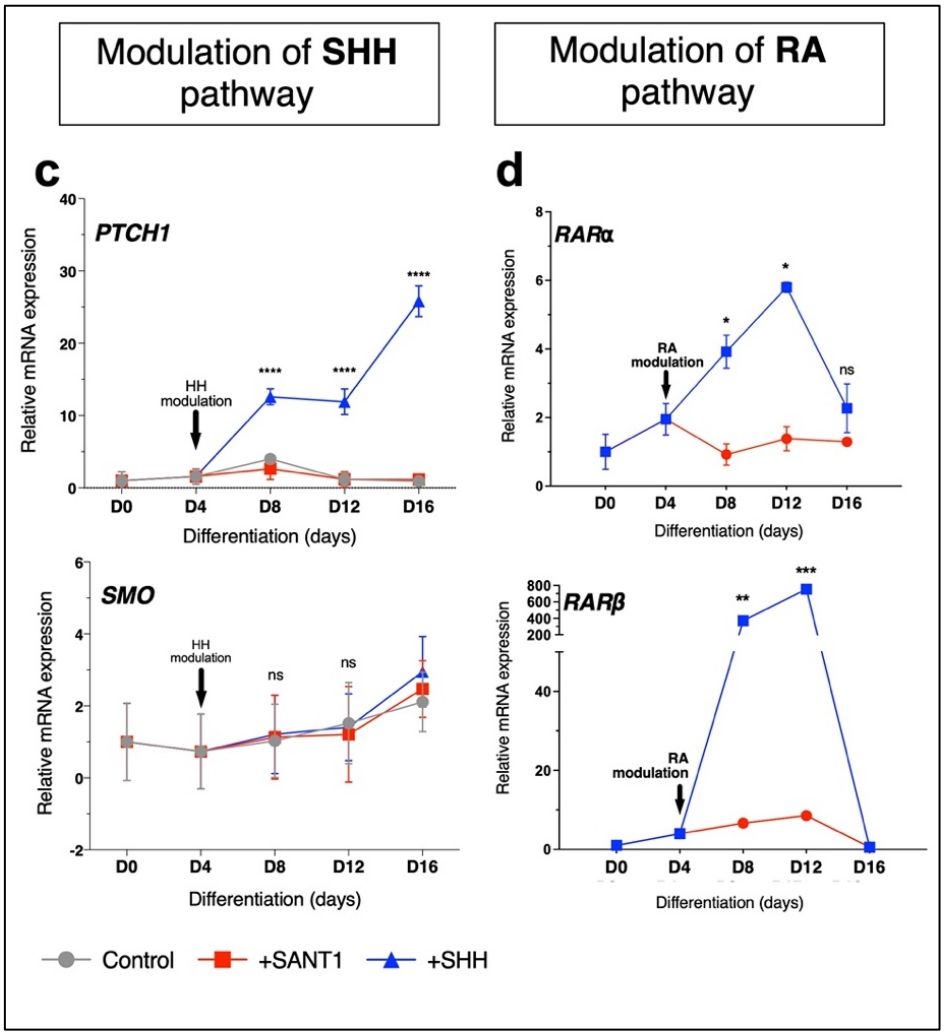
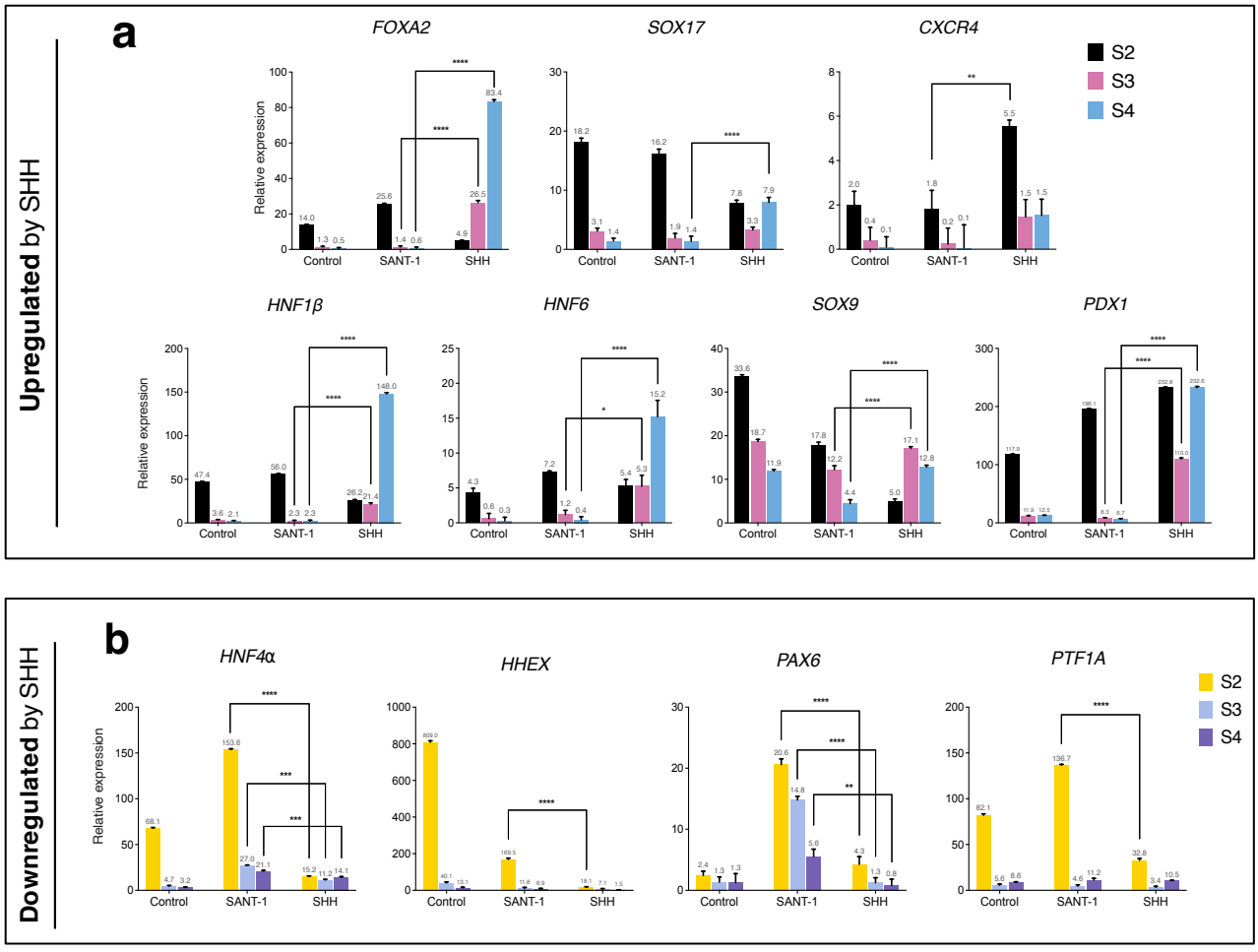


Figure 3: *In vitro* modulation of the sonic hedgehog and retinoic acid pathways alter the expression of their known target genes.
(**a**) Protein levels of PTCH1 and GLI1, SHH, and SMO.
(**b**) Bar graphs represent quantitation of proteins normalized to β-ACTIN.
(**c**) mRNA levels of *PTCH1* and *SMO* in response to SHH modulation.
(**d**) mRNA levels of RARα and RARβ in response to RA modulation.
*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

iv. **Effect of SHH and RA pathway modulation on pancreas differentiation -** We observed that at stage 2 when modulation of the pathway had just begun, no drastic changes occurred in the expression of most transcription factors. However, on prolonged treatment with SHH modulators expression of key genes such as *FOXA2*, *SOX17*, *HNF1β*, *HNF6*, *SOX9*, and PDX1 were altered between the groups. Treatment of the differentiating cells with exogenous SHH led to several fold increase in *FOXA2*, *SOX17*, *HNF6*, and *SOX9*. Levels of *HNF1β* and *PDX1* increased nearly 150 to 200-fold. Comparatively, expression of these markers remained low in inhibitor-treated cells. This increase was also confirmed at the

protein levels for PDX1. Interestingly, expression of these transcription factors increased at stage 3 and remained high through stage 4 even after withdrawal of SHH. These markers are involved in development of the ductal system and sustained expression of these markers is seen in progenitor cells that give rise to the pancreatobiliary system. More importantly, an active SHH pathway repressed the expression of markers involved in pancreatic endoderm formation i.e., *HNF4α*, *HHEX*, *PAX6*, and *PTF1A*. Markers HNF4α and PAX6 regulate endocrine specification, while PTF1A and HHEX are involved in acinar and ventral pancreas formation respectively (see Figure 4).



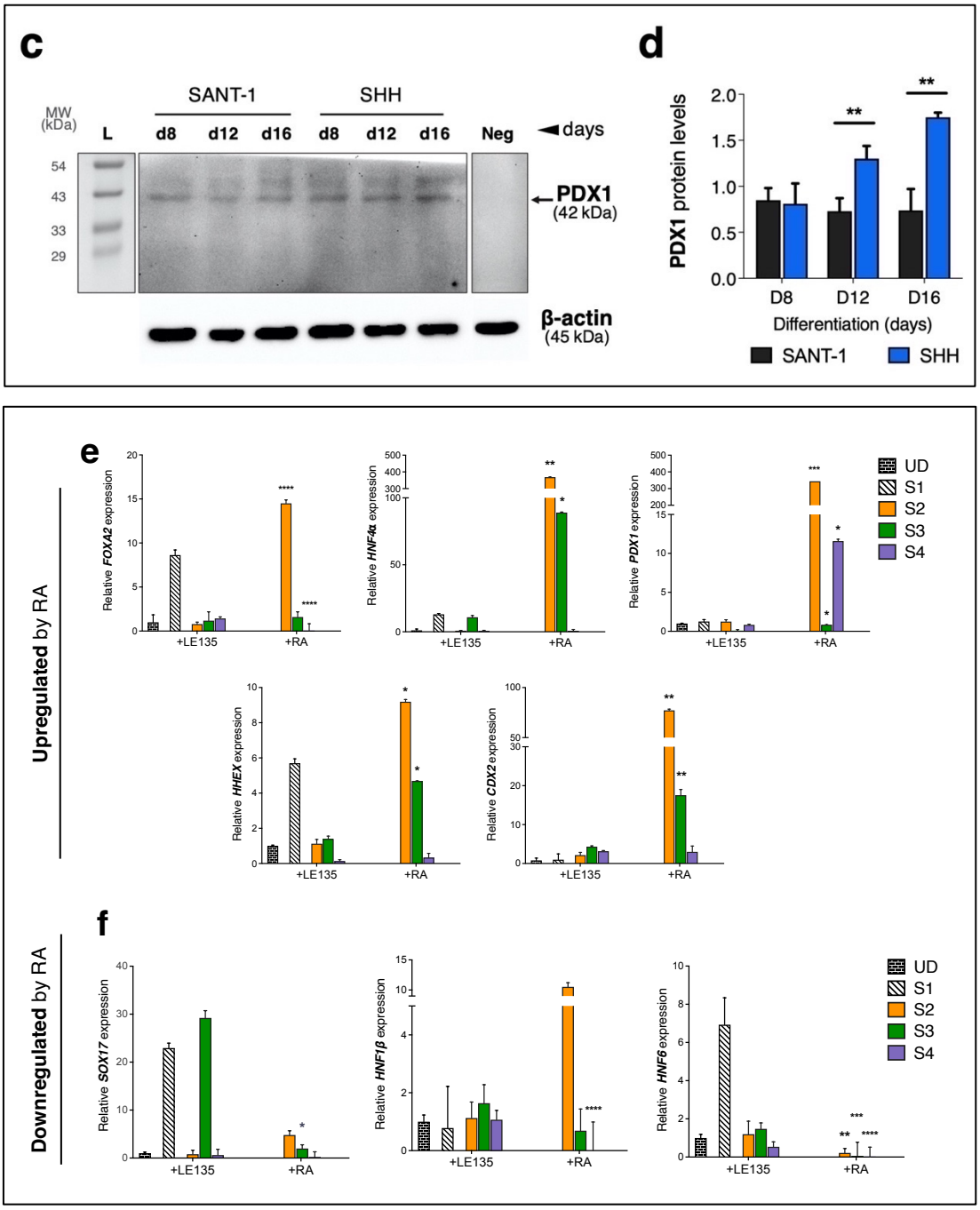


Figure 4: SHH and RA modulation during pancreatic differentiation alters transcription signatures at posterior foregut and pancreatic endoderm stages.

mRNA levels of specific markers that responded to SHH modulation *in vitro*, measured by quantitative reverse-transcription PCR. Activation of the SHH pathway leads to an upregulation of markers *FOXA2*, *SOX17*, *CXCR4*, *HNF1β*, *HNF6*, *SOX9*, and *PDX1* (**a**) while, the addition decreases *HNF4α*, *HHEX*, *PAX6*, and *PTF1A* expression (**b**). mRNA levels were normalized to house-keeping, *18S rRNA*, and expression was plotted relative to levels in undifferentiated cells. Error bars denote mean ± s.e.m. from three technical repeats of three independent biological studies each (n=3). *P* values computed using Two-Way ANOVA followed by Tukey's multiple comparisons test. Asterisks denote *P* values and represent significance between SANT1 /SHH treatments at specific time points /stages. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

(**c**) Immunoblot analysis showing PDX1 expression upon sonic hedgehog modulation.
(**d**) Bar graphs represent quantitation of PDX1 protein normalized to β-ACTIN.
(**e**) Activation of the RA pathway leads to an upregulation of markers *FOXA2*, *HNF4α*, *HHEX*, *CDX2*, and *PDX1* while, the addition decreases *SOX17*, *HNF1β*, and *HNF6* expression (**f**). mRNA levels were normalized to house-keeping, *18S rRNA*, and expression was plotted relative to levels in undifferentiated cells.

v. **Effect of SHH and RA pathway modulation on Polycomb subunits in the pancreatic progenitors -** Since both, SHH and BMI1/PRC1, are known to play crucial roles in pancreatic development, we hypothesized that the downstream molecules of SHH pathway were capable of interacting with and regulating PcG subunits during pancreatic development. To assess this, we analyzed levels of PRC1 subunits in the differentially treated progenitors. We found *BMI1* was overexpressed when the SHH pathway was stimulated as compared to groups which were treated with SANT1 that displayed lower levels of BMI1 (Figure 5). A similar pattern was seen at the protein levels wherein levels of BMI1 were higher in agonist treated groups as compared to control, demonstrating BMI1 can respond to varying levels of SHH signals. Remarkably, BMI1 levels remained higher in SHH-treated cells at stages even after the agonist was removed from the culture media. Our data suggests that SHH can regulate BMI1 during pancreatic differentiation from hESCs. As RING1B/BMI1 form the core catalytic subunits of Polycomb Repressive Complex 1 that performs histone PTMs, we next examined whether increased BMI1 levels correlated with their histone marks. To this end, we extracted histones from cells subjected to a modulated SHH pathway and performed anti-H2AK119ub1 immunoblotting. Total histone H2A protein was used as loading control and for semi-quantitation of the immunoblots. We observed no changes in the global levels of H2AK119ub1 in the pancreatic progenitors as the HH pathway was modulated. *In vivo* PcG components display dynamic behavior allowing them to regulate the chromatin in a transient fashion. Thus, suggesting that repression in pancreatic progenitors is primarily based on distribution along gene promoters rather than an overall expression. This finding made it necessary for us to check whether PRC1 components are enriched at key pancreatic genes upon sonic hedgehog pathway modulation.

BMI1 and *RING1B* levels showed significant differences in cells with a modulated retinoic acid pathway. Notably, we observed a significant decrease in *RING1B* expression on prolonged exposure to retinoic acid. No such decrease was seen in RA-inhibitor treated

cells. However, surprisingly, RA induced expression of *BMI1*, levels of which stayed high until the pancreatic endoderm stage (see Figure 5).

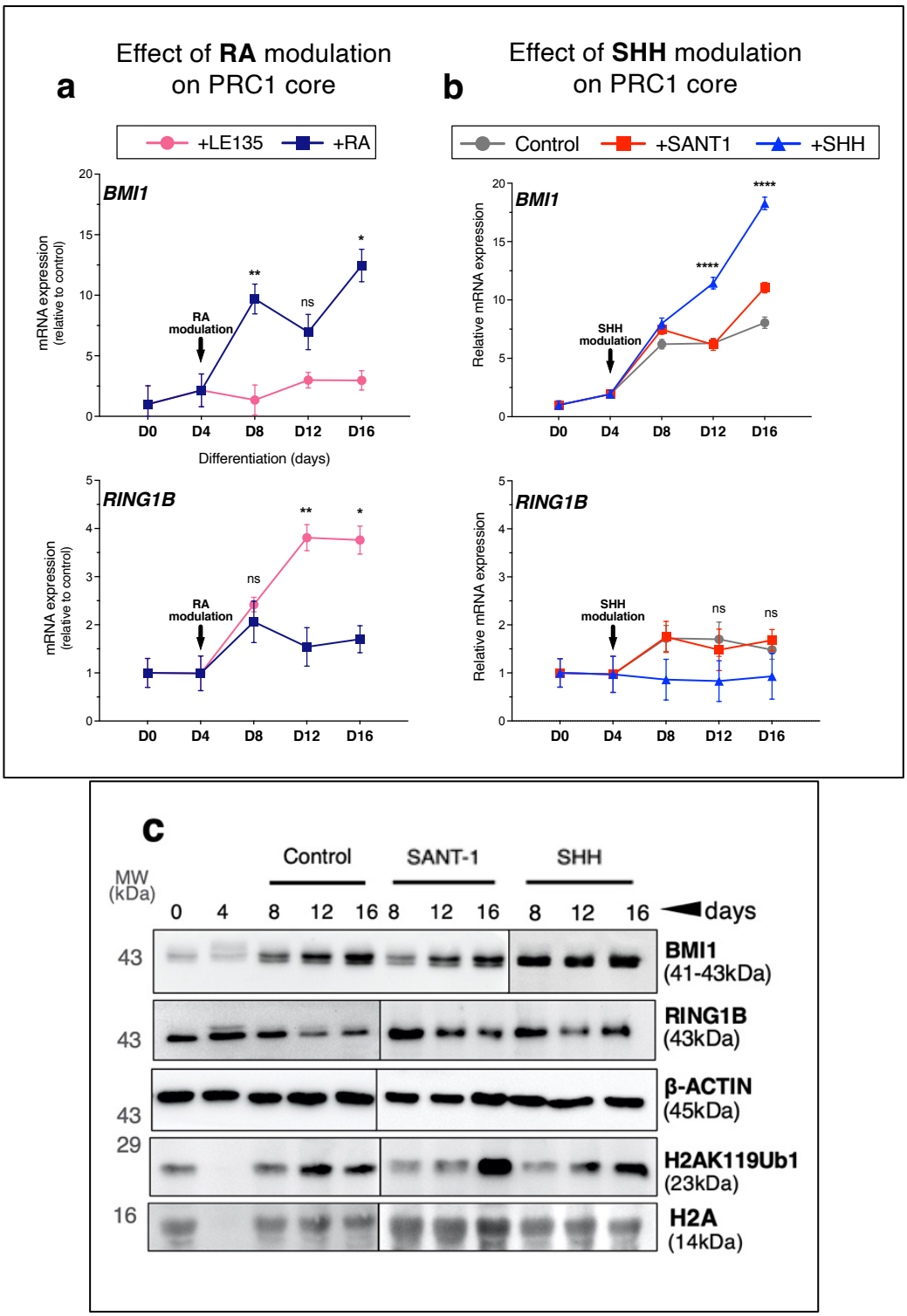
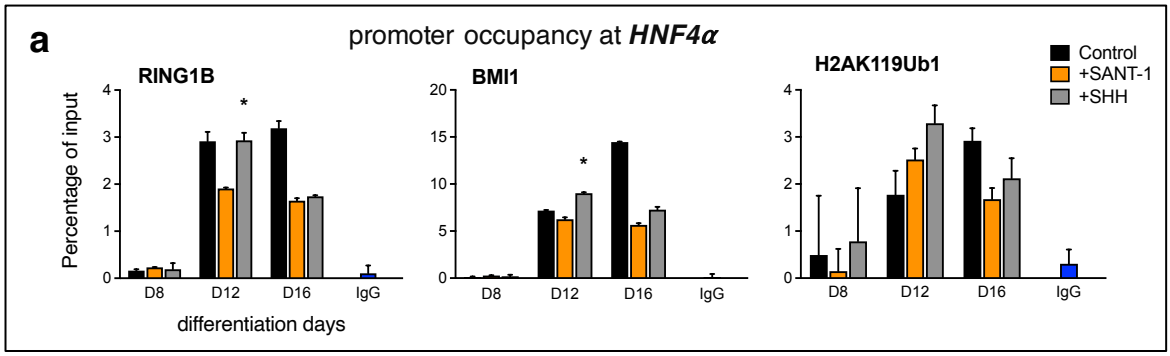
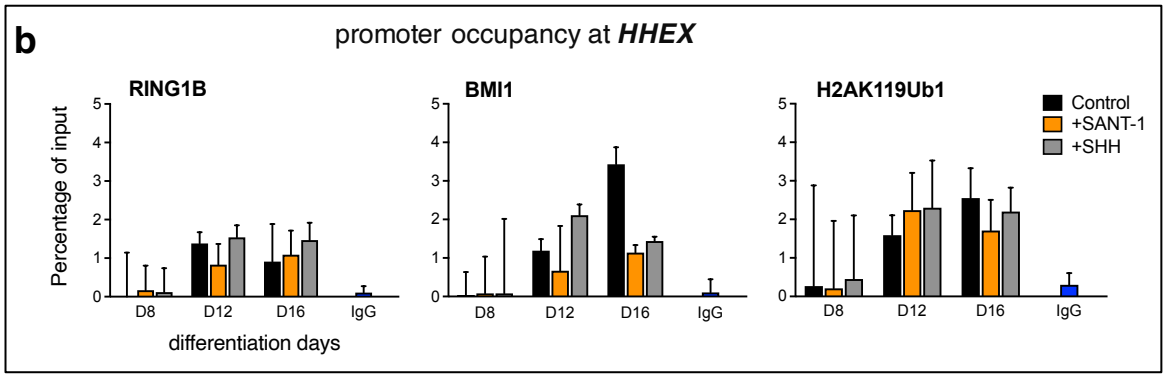


Figure 5: Levels of PRC 1 core subunits in response to SHH and RA modulation. Polycomb group proteins, *BMI1* and *RING1B* mRNA expression in cells, differentiated in the presence of RA modulators (**a**) and SHH modulators (**b**). Transcript levels were analyzed by qRT-PCR with mRNA levels normalized to house-keeping, *18S rRNA* and expression was plotted relative to levels in undifferentiated cells. Error bars denote

mean±s.e.m from three technical repeats of three independent biological studies each (n=3). *P* values computed using Student's t-test. Asterisks denote P values and represent significance between SANT1 /SHH or LE135 /RA treatments at specific time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.
(**c**) Levels of Polycomb Repressive Complex 1 core proteins and its mark measured using anti-BMI1, anti-RING1B and anti-ubiquityl-Histone H2A (Lys119) immunoblots in the differentiated cells. Expression of BMI1 and RING1B proteins was normalized to β-ACTIN and expression of H2AK119ub1 protein was normalized to total H2A. Immunoblot studies were performed in three technical repeats from two independent biological studies each (n=2).

vi. **Analysis of PcG occupancy at pancreas-specific promoters in response to SHH and RA modulation -** To check whether increased BMI1/RING1B was catalytically active at these gene promoters, we also performed ChIP-qPCR for H2AK119ub1. Interestingly, we observed increased binding of BMI1 at the *HNF4α* promoter upon SHH addition at days 12 and 16 (Figure 6). No such increase was seen in the groups treated with the pathway inhibitor. At the promoter of *HNF4α*, enrichment of BMI1 also corresponded to higher H2AK119ub1 levels. At the promoters of *HNF1β* and *SOX9* we observed significant occupancy of BMI1 and RING1B upon addition of exogenous SHH. However, no such increase was seen in the PRC1 histone modification, H2AK119ub1. We found low binding of BMI1 at the pancreas crucial markers, *PDX1* and *SOX17* throughout the differentiation days. In all the genes, RING1B occupancy at day 12 remained lower in the SANT1 treated groups. The liver-specific marker, *ALB*, was also enriched with BMI1 at day 12 in the SHH-treated cells. Interestingly, barring *SOX17* and *PDX1*, promoters of genes with varied functions across pancreas development showed increased binding of BMI1 when the SHH pathway was activated in the progenitor cells (see Figure 6).

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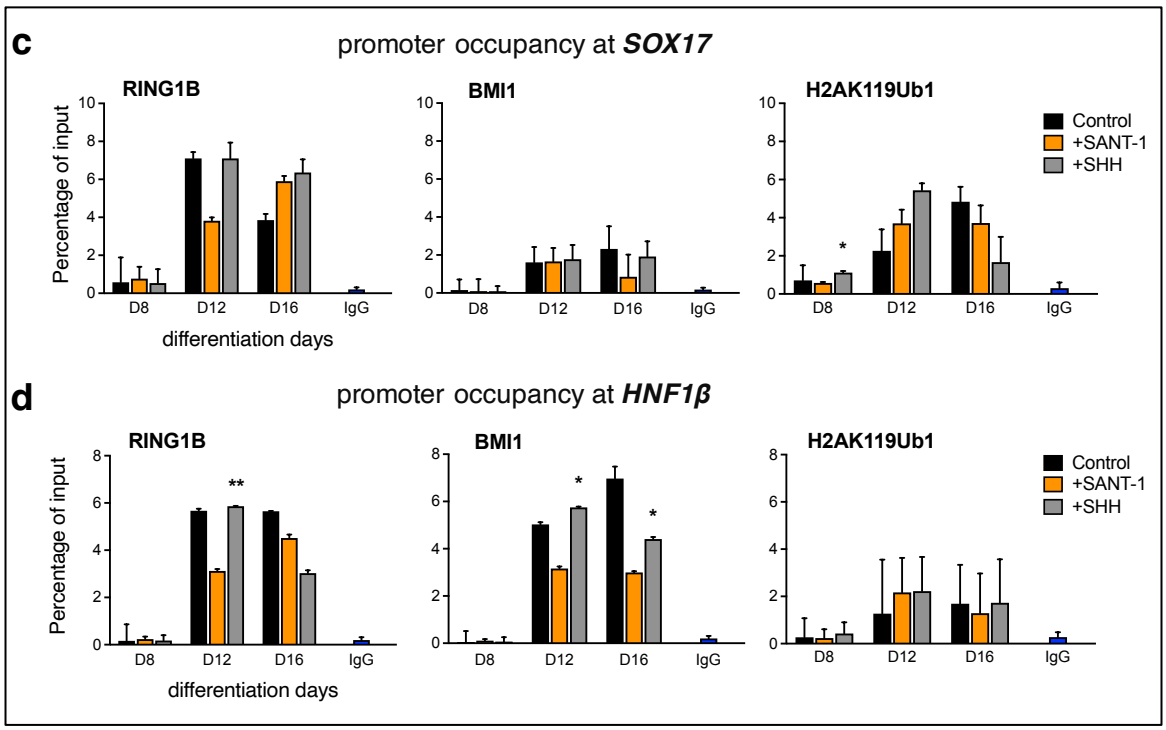
promoter occupancy at HHEX
RING1B
BMI1
H2AK119Ub1
Control
+SANT-1
+SHH
Percentage of input
D8
D12
D16
IgG
differentiation days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RING1B
BMI1
H2AK119Ub1
Control
+SANT-1
+SHH
Percentage of input
D8
D12
D16
IgG
differentiation days
d
promoter occupancy at HNF1β
RING1B
BMI1
H2AK119Ub1
Control
+SANT-1
+SHH
Percentage of input
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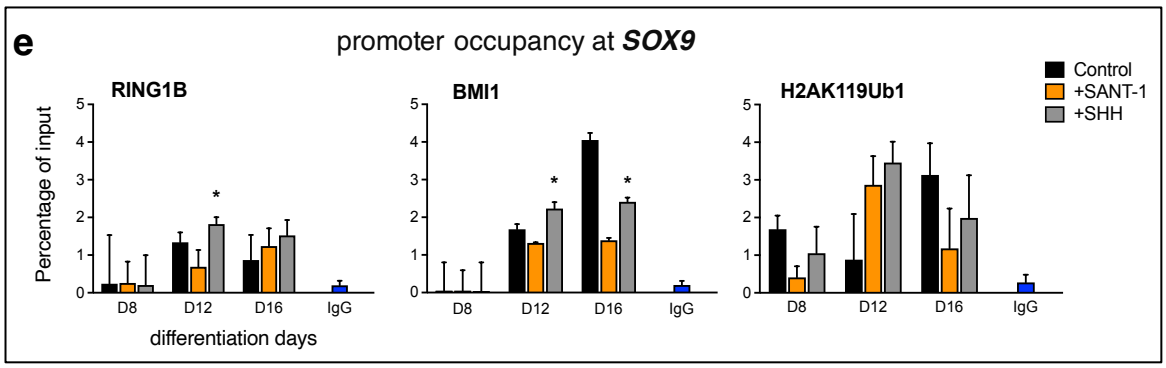
IgG
differentiation days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Percentage of input
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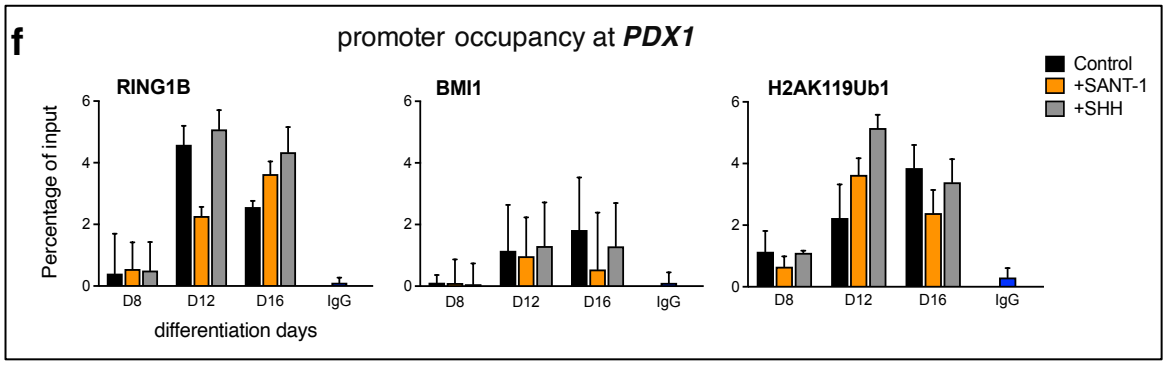
differentiation days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Percentage of input
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D12
D16
IgG
differentiation days

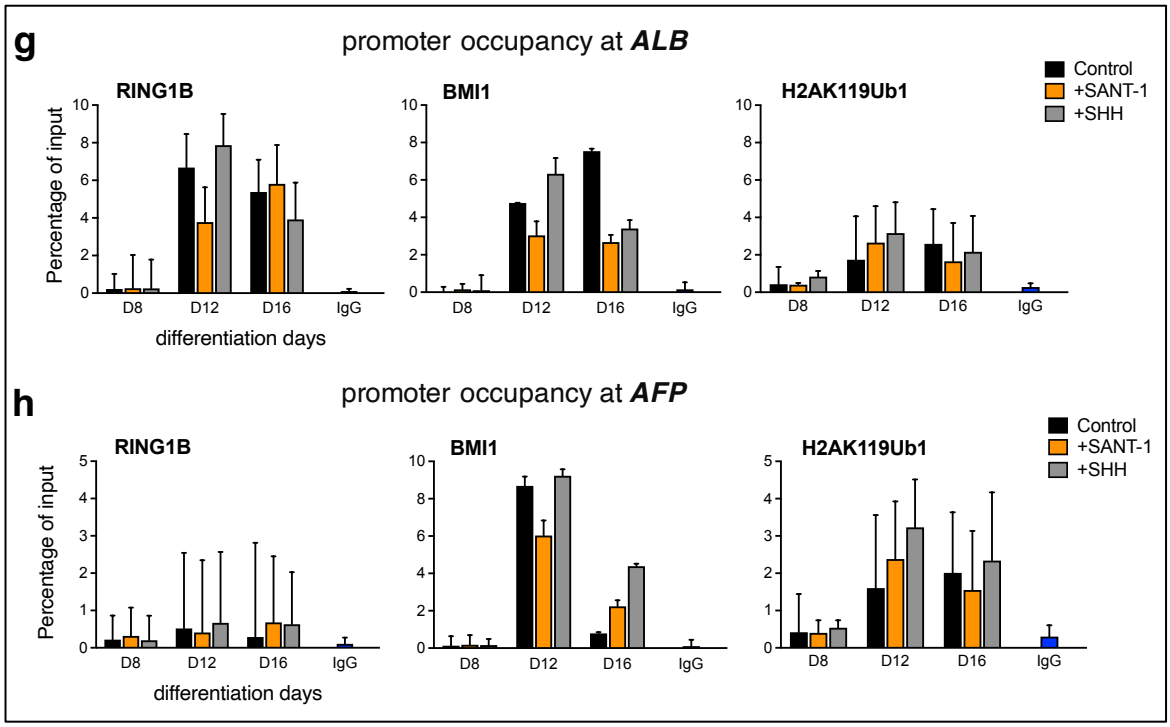


Figure 6: Chromatin immunoprecipitation coupled with quantitative PCR.
ChIP-qPCR for PRC1 core subunits RING1B, BMI1, and PRC1 mark H2AK119ub1 at the promoters of pancreatic specific genes during modulation of the sonic hedgehog pathway in pancreatic progenitors. Occupancy studied at the promoters of *HNF4α* (**a**), *HHEX* (**b**), *SOX17* (**c**), *HNF1β* (**d**), *SOX9* (**e**), *PDX1* (**f**), *ALB* (**g**), and *AFP* (**h**). Error bars denote mean±s.e.m from three technical repeats of two independent biological studies each (n=2). *P* values computed using Student's t-test. Asterisks denote *P* values and represent significance between SANT1/SHH treatments at specific time points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant with $p > 0.05$.

DISCUSSION:

In this work, we set out to determine the role and molecular mechanism of sonic hedgehog signals during the development of pancreatic progenitor cells. Our data demonstrates that during human embryonic stem cell differentiation into pancreatic lineage, sonic hedgehog (Shh) signals impede pancreatic differentiation by suppressing gene expression patterns necessary for pancreatic epithelial formation and shh signals promoted pancreatobiliary cell fate. We uncovered the coordination between SHH and RA pathways and PRC1 proteins during specification of pancreatic endoderm cells, axes which were poorly understood previously. Although several groups have efficiently converted hESCs into pancreatic progenitor cells *in vitro* using morphogens/cytokines/growth factors, very little research is done on the molecular associations between signaling pathways and histone modifiers, especially PcG proteins.

We found that lineage transition from the definitive endoderm to posterior foregut stages was accompanied by distinct patterns of genes, which varied widely across the treatment groups. Exogenous activation of SHH pathway in the progenitor cells led to continued higher expression of *FOXA2*, *SOX17*, and *CXCR4* even at posterior foregut and pancreatic endoderm stages as compared to control and inhibitor-treated cells. The ventral pancreas and the biliary system share a common progenitor pool in the caudal regions of the ventral foregut where, Sox17 is necessary for and sufficient to induce ductal fate and specifies organ boundaries by restricting expression of Pdx1 in the progenitor populations (Spence *et al.,* 2009). Concomitantly, loss of Sox17 leads to the formation of ectopic pancreas in the common duct region, suggesting progressive silencing of Sox17 in stages post definitive endoderm is a requirement for the development of the pancreatic domain. Therefore, our data concurred with published literature and demonstrated that upregulation of *SOX17* upon addition of SHH leads to a pancreatobiliary fate. In contrast to inhibitor-treated studies, prolonged exposure to exogenous recombinant SHH upregulated the expression of *HNF1β*, *HNF6*, PDX1, and *SOX9*; all of which are critical determinants involved in ductal morphogenesis and formation (Pierreux *et al.,* 2006; Wescott *et al.,* 2009; Shih *et al.,* 2012; Quilichini *et al.,* 2019). In mice, during early pancreatic development, colocalization of Hnf6 is seen with Pdx1 throughout the pancreatic epithelium. However, at later stages, Hnf6 is restricted to ductal cells with minimal expression in acinar and endocrine domains (Pierreux *et al.,* 2006). Similarly, studies by Solar *et al.* (2009) have shown that early Hnf1β+ cells are precursors of endocrine, exocrine, and ductal lineages. During later stages

of mice pancreas development, expression of this transcription factor persists only in duct cells. A surge in *HNF1β* and PDX1 could also be attributed to the fact that they are transcriptional targets of a network governed by SOX17 and FOXA2, respectively (Lee *et al.,* 2019). Our results also correlate to studies in 3D culture, wherein branching cells of the ductal pancreas transiently express high levels of PDX1. Gu *et al.* (2002) clearly show that all types of pancreatic progenitors express Pdx1 during the early stages of embryogenesis; however, only cells expressing Pdx1 at later stages of development give rise to pancreatic duct cells.

Excluding SHH signals from the differentiating cells through pathway antagonist SANT1 resulted in several fold increase of *HNF4α*, *HHEX*, *PTF1A*, and *PAX6* –genes essential for pancreatic epithelium specification. HNF4α controls gene regulatory networks involving critical functions of glucose metabolism and insulin secretion in β-cell (Wang *et al.,* 2000). Haematopoietically expressed homeobox (Hhex) is involved in the initial specification of the ventral pancreatic program with Hhex-null mice embryos showing a complete absence of PDX1 and subsequently the ventral pancreas bud (Bort *et al.,* 2004). Ptf1A factor is enriched in mice multipotent progenitors destined to become pancreatic epithelium (Zhou *et al.,* 2007). As differentiation ensues, expression of Ptf1A becomes restricted almost exclusively in acinar cells with silencing of this gene in cells destined to become endocrine or ductal (Masui *et al.,* 2007). Paired-box homeodomain 6 (Pax6) controls transcription of genes critical for endocrine functions. Its early expression in mice restricted to pancreatic progenitors that eventually give rise to mature endocrine cells (St-Onge *et al.,* 1997; Swisa *et al.,* 2017). Both Pax6 and Hhex contribute towards islet organization and maintenance of islet cell phenotypes, with mutations in these genes affecting endocrine differentiation (Zhang *et al.,* 2014; Mitchell *et al.,* 2017).

We find that the outcome of modulating sonic hedgehog signals during differentiation is not a binary result, but rather like all developmentally crucial morphogens, it produces a variety of cell types from a shared pancreatic progenitor pool. With almost all differentiation protocols generating pancreatic progenitors from hESCs incorporate SHH inhibitors at near same concentrations of 0.25 μM. We show why it is necessary to suppress the sonic hedgehog pathway during pancreatic differentiation from hESCs. We also maintained the concentration of inhibitor used by most research groups, and it may not completely shut down the SHH pathway. Unlike genetic manipulation of SHH or SHH receptor genes, the subtle changes induced by the use of inhibitor might be close *to in vivo* conditions, where gradients form as a micro-scale. Although the morphological consequences of ectopic

activation of Shh during late pancreatic development in mice have been described earlier (Kawahira *et al.,* 2005), our study is the first to report the transcriptional network governed by sonic hedgehog during *in vitro* pancreatic differentiation from hESCs. Despite many similarities between mice and human pancreas development, sonic hedgehog morphogens have varying roles across species, necessitating investigation of the subtle differences present (Folias & Hebrok, 2014). Given the role retinoic acid plays in pancreas development in vivo, we used small molecule inhibitor of RA and all-trans retinoic acid during directed differentiation of hESCs. Modulation of the retinoic acid allowed us to understand the transcriptional network governed by RA signals during pancreatic differentiation. Addition of RA upregulated several genes required for pancreas specification - *FOXA2*, *HNF4α*, *PDX1*, *HHEX*, and *CDX2*. Previous reports show exposure to RA induces removal of PRC repressive mark, H3K27me3 from Hox gene clusters (Kashyap *et al.,* 2011). Since, treatment with all-trans RA is known to decrease binding of RING1B and H3K27me3 during mouse development (Yakushiji-Kaminatsui *et al.,* 2018), we examined the expression of PRC1 components in the pancreatic progenitors upon modulation of RA signals. Concordantly, we found that activation of the RA pathway led to a decrease in *RING1B* as compared to when the pathway was inhibited. It is possible that the reduction in RING1B allows transcriptional activation of *FOXA2*, *HNF4α*, *PDX1*, *HHEX*, and *CDX2* by retinoic acid. Intriguingly, active an active RA pathway increases expression of PRC1 subunit BMI1. No such induction in the LE135-treated cells indicate that BMI1 could be a downstream target gene of the RA pathway in the progenitor cells.

To understand this repressive role played by SHH, we examined Polycomb group proteins in our treated hESCs. As PcG proteins allow for fine-tuning of heritable changes of developmental genes in response to extracellular cues, we wanted to examine their expression upon sonic hedgehog signal modulation. Upregulation of BMI1 in pancreatic progenitors treated with exogenous SHH suggested a positive induction of the epigenetic modulator BMI1 by sonic hedgehog morphogens during pancreatic specification. No such increase was seen in inhibitor-treated cells. Why then do levels of the subunit catalyzing the histone post-translational modification not change? Although RING1B inherently contains the E3-ligase domain necessary for adding PRC1-mediated marks, binding of BMI1 to RING1B is essential and sufficient for stimulating this enzymatic activity (Li *et al.,* 2006). Increased levels of BMI1 upon the addition of exogenous SHH could be responsible for augmenting PRC1-mediated ubiquitination without actually increasing global RING1B levels, this could answer why global RING1B levels do not change upon modulation.

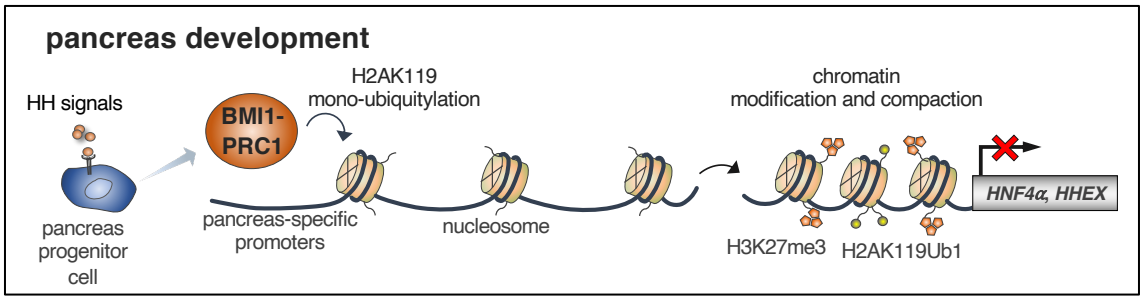
We next tested occupancy of PRC1 (RING1B and BMI1) to pancreas-specific promoters in our in vitro system. Coupling of ChIP with qPCR gave us a dynamic view of the PRC1 subunits and their mark during cell fate transitions from definitive endoderm to pancreatic progenitors. Prior studies had mainly focused on the H3K27me3 and H2AK119ub1 marks (Arensbergen *et al.,* 2012; Xie *et al.,* 2013), but analysis of the Polycomb group subunits at intermediatory stages of pancreas differentiation had not been performed. Upon activation of SHH signals, we found a significant increase in the binding of BMI1 at the promoters of pancreas-specific markers. The presence of very low H2AK119ub1 marks at *ALB* and *PDX1* in the progenitors suggest these loci could be regulated by PRC2 or by mechanisms other than PcG proteins. Since *ALB* expression is suppressed in late-stage pancreatic progenitors, it is possible that DNA methylation or more stringent epigenetics modifiers are at play here. The distal promoter regions of *PDX1* have several CpG sites that display high levels of DNA methylation (Yang *et al.,* 2012). This could explain why cells treated with SHH addition display higher binding of RING1B and H2AK119ub1 but also show several folds higher *PDX1* transcript expression. Surprisingly, SHH did not alter BMI1 levels at *PDX1* promoter as compared to the other genes analyzed. *HNF4α*, *HHEX*, *HNF1β*, *SOX9*, *AFP*, and *ALB*, all displayed increased promoter occupancy by BMI1 at days 12 and 16 upon treatment with exogenous SHH.

In summary, our studies revealed the transcription network governed by sonic hedgehog and retinoic acid signals during the differentiation of hESCs into a pancreatic lineage. Modulation of SHH and RA signals altered levels of PcG subunits in the differentiating cells. We demonstrate the restrictive role of sonic hedgehog towards pancreatic endoderm formation while promoting pancreatobiliary fates during differentiation of human pluripotent stem cells. Our results explain why several differentiation protocols utilize SHH inhibitors and RA agonists during the specification of β-cells. We now show, consistent with previous reports, that sonic hedgehog coordinates with epigenetic complexes to affect the transcription of developmental regulators essential for pancreas formation.

Given that Hh signals elicit their responses as morphogens and regulate Bmi1 in a dosage-dependent manner (Fukuda *et al.,* 2012), it would be interesting to see how varying concentrations of antagonist /agonist could affect the cell fate choices in multipotent progenitors. It would also be interesting to study the effect of conditional knockouts of PRC1 subunits containing histone ubiquityl activity or inhibiting catalytic activity of PRC1 using RING1B/ BMI1 inhibitors. Such studies would provide further details into the role of

PRC1 in pancreas development. The present study has elucidated the associations between PRC1 and how they are regulated at pancreas-specific genes by extracellular signals.

CONCLUSION/ SUMMARY:



- Modulation of SHH and RA pathways in the differentiating cells altered the expression of PRC1 core subunits BMI1 and RING1B in pancreatic progenitors
- Activation of SHH pathway promoted pancreatobiliary fates while inhibition induced formation of pancreatic endoderm
- BMI1 occupied promoters of *HHEX*, *HNF4α*, *AFP*, and *ALB* upon stimulation by SHH signals
- Promoters of key genes *PDX1*, *SOX9*, and *SOX17* appear to be regulated by epigenetic factors other than PRC1
- Modulation of the retinoic acid pathway affected several of the pancreas-specific transcription factors, regulating the fate of the differentiating cells
- Levels of BMI1 and RING1B were altered upon modulating the RA pathway, suggests that retinoic acid can cooperate with PcG proteins to mediate pancreatic differentiation
- Overall, our study shows the molecular mechanisms by which SHH and RA affect pancreatic differentiation form human embryonic stem cells

SIGNIFICANCE OF THE STUDY:

Our study exemplifies how the interplay between sonic hedgehog and Polycomb group proteins (PcG) regulate the complex patterning and formation of the pancreas during differentiation of human pluripotent stem cells. The transcriptional network governed by retinoic acid during hESCs derived-pancreatic progenitors was revealed. Understanding the mechanisms regulating PcG proteins could improve our comprehension of human pancreatic progenitor formation.

PUBLICATIONS AND CONFERENCES:

- **Dumasia, N.**, Khanna, A., & Pethe, P. (2021). Sonic hedgehog signals hinder the transcriptional network necessary for pancreatic endoderm formation from human embryonic stem cells. Genes To Cells. https://doi.10.1111/gtc.12839
- Pethe, P., **Dumasia, N.**, & Bhartiya, D. (2021). Effect of Sonic hedgehog pathway inhibition on PDX1 expression during pancreatic differentiation of human embryonic stem cells. Molecular Biology Reports. https://doi.10.1007/s11033-021-06147-w
- **Dumasia, N.**, & Pethe, P. (2020). Pancreas development and the Polycomb group protein complexes. Mechanisms Of Development, 164, 103647. https://doi.10.1016/j.mod.2020.103647
- Desai, D., **Dumasia, N.**, & Pethe, P. (2017). Polycomb Group Proteins: Emerging Players in Neurogenesis. Biomedical Research Journal, 4(2), 122-136.
- Manuscript entitled 'Regulation of Polycomb group proteins by small molecules: Implications in stem cell biology' ready for submission.
- Manuscript entitled 'Polycomb protein BMI1 associates with Sonic hedgehog signaling during human embryonic stem cells derived pancreatic progenitors' ready for submission.

Conferences:

- **EMBL Conference: Chromatin and Epigenetics. 17-20 May, 2021.** Presented poster titled 'Sonic hedgehog morphogens cooperate with Polycomb group protein BMI1 to regulate pancreatic differentiation'.
- **International Society for Stem Cell Research ISSCR 2018 Annual Meeting. 20-23 June 2018, Melbourne, Australia.** Presented poster titled 'Emerging alliances: signaling pathways regulate histone modifiers during pancreatic differentiation of human pluripotent stem cells'.

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